

**Transfer of eDNA sample processing capabilities from US Army Corps of Engineers,
Engineering and Research Development Center's Center for eDNA Application and Research
(ERDC-CEDAR) to the Whitney Genetics laboratory (WGL),
US Fish and Wildlife Service**

Transition Plan Report

August 1, 2013



**US Army Corps
of Engineers®**

eDNA Transition Team signatures: Our signatures indicate that we believe the Whitney Genetics Lab has successfully completed the transition plan and demonstrated competence in executing the QAPP in analysis of eDNA samples.

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General Summary

This report includes results from transition sample analysis laid out in the Transition Plan (plan) which was approved 21 Dec 2012 by Region 3 Deputy Regional Director, Charles Wooley. The plan was developed by the eDNA Transition Working Group (USACE and USFWS representatives), and outlines the tasks to be executed by the Whitney Genetics Laboratory (WGL) of the USFWS and the Engineering and Research Development Center's Center for eDNA Application and Research (ERDC-CEDAR) of the USACE, in order to complete the transition of eDNA processing capacity from USACE to USFWS. This report will only cover results regarding tasks 1.2 to 1.4 explained on pages 9-12 of the plan, but includes a description of brief methods and passing criteria for each task from the plan. This report also includes results from additional analyses carried out in support of ECALS work, only as further demonstration of WGL abilities to process samples, not to provide any interpretation on the outcome of the ECALS studies.

At the time the plan was approved, all positions had been staffed, the new WGL building had been occupied and FY 2012 funds had been used to procure equipment and supplies. FY 2013 funds have also been resourced (around \$620K) to add additional equipment and supplies as work on the transition samples proceeded. Examples of new equipment include an Applied Biosystems Genetic analyzer, BioRad laser imaging system, and 8 BioRad dual-purpose thermocyclers.

The Laboratory Information Management System (LIMS) was installed for training the week of 13 May 2013. During the next month, Emy Monroe (EMM) will take LIMS configuration/manager web-ex training to build the system and determine what customizations are needed for WGL, followed by modification of the software. Finally, the functioning system will be installed, tested, and a one-week end-user training session will occur at WGL. As of 17 May 2013, the STARLiMS trainer estimated about two months until the LIMS would be fully operational. Therefore, the eDNA Transition Working Group recommends that WGL begin processing samples once we are approved via the plan, even before the LIMS is activated. WGL will follow the QAPP and keep data books and electronic files of results until the LIMS is active. Once LIMS is active, the hard-copy records and electronic records will be archived and preserved at WGL.

Training and practice kit processing occurred between 7-13 January 2013 and each analyst processed as many practice kits as needed until quality control samples were as expected.

Overview of transition tasks:

Task 1.1. Samples (35) were collected from Lake Calumet in the CAWS, filters were cut in half and divided between CEDAR and WGL labs. WGL results were within 50% of CEDAR results, therefore WGL passed this task.

Task 1.2. Archived samples (120) were sent from ERDC-CEDAR to WGL. Each WGL staff member analyzed 20 samples and compared results to original monitoring results obtained at ERDC-CEDAR, and 68 of 120 samples had the same results between labs, which did not meet the plan criterion. This task was repeated during the transition visit, where ERDC-CEDAR and WGL analysts ran 30 samples side-by-

side in cPCR and qPCR and results were within 20% agreement for cPCR and 10% agreement for qPCR, therefore WGL passed this task.

Task 1.3. Lab-prepared blind water samples were prepared by EMM, and each analyst processed 20 filters that included 5 replicate blanks, 5 silver carp positives, 5 bighead carp positives, and 5 samples from 5 non-bigheaded carp fish species. Three analysts obtained passing results on the first kit, one analyst processed four kits, and the last analyst processed three kits in order to achieve passing results.

Task 1.4. Field samples from Lake Neshonoc, WI (20) and the lower Illinois River, IL (20) were collected and filtered according to the QAPP. Samples were processed according to the QAPP except that filters were extracted with the Qiagen kit instead of the PowerWater kit. There were 0 confirmed positive samples from Lake Neshonoc for either carp species, and 9 confirmed positive for silver and 0 positive for bighead from the lower Illinois River. These results met the plan criterion, therefore WGL passed this task.

Additional studies to demonstrate lab ability to process eDNA samples with quality control results as expected:

1. WGL filtered (according to the QAPP) 360 field samples that had been spiked with silver carp DNA. WGL then analyzed 120 samples (several filters per sample) in the lab, half extracted with the Qiagen kit and half extracted with the PowerWater kit. Extracts were amplified for silver carp DNA according the QAPP (cPCR) as well as by quantitative PCR (qPCR) according to the study protocol. WGL results were within 25% agreement with USGS's Upper Midwest Environmental Science Center lab results for cPCR and qPCR across both extraction methods.
2. WGL processed 100 field samples, 20 blanks and 80 that had been spiked with silver carp DNA with a centrifugation technique instead of filtering to concentrate the DNA. The samples were extracted with the Qiagen kit, and the QAPP was followed for remaining analyses. All quality control samples were as expected, and samples amplified as expected in 94 of 120, and were within 20% agreement with WGL results from the same treatments that had been filtered.

The transition team visited the WGL 11-14 June 2013 to see the facility, observe sample processing and check out procedures and adherence to the QAPP. ERDC-CEDAR provided some feedback and WGL has responded to them in this report.

WGL staff has "passed" 4 of 4 tasks outlined in the transition plan, as well as in two additional eDNA studies. Therefore, the eDNA Transition Working Group recommends WGL analysts and the lab are ready to process samples. Overall, a lack of contamination issues in field and lab negative controls indicate the lab is successful at preventing contamination despite handling large amounts of water containing carp DNA and PCR products containing millions of copies of DNA.

Transition Methods, Results and Discussion

Methods:

The transition plan tasks and results are outlined below. In tasks 1.1, 1.3, and 1.4, water samples were filtered and WGL staff processed filters according to the QAPP unless otherwise stated. Task 1.2 used archived samples, so there was no extraction step. In tasks where filters were extracted, positive and negative extraction controls were made by the following method. Pure silver carp or pure bighead carp cell lines were obtained from the La Crosse Fish Health Center virology lab. Cell solutions were divided into aliquots of ~500 ml and DNA was extracted in the Fish Health PCR area (to avoid contaminating WGL) with Qiagen DNeasy blood and tissue kits, producing a final volume of 200 µl. One aliquot of bighead and one aliquot of silver carp DNA extract were added to 49 ml of sterile molecular grade water in a sterile 50-ml centrifuge tube. The mixture was inverted several times. Sterile filters were folded and placed into 1.5-ml microcentrifuge tubes and 200 µl of the diluted extracts were pipetted onto each filter. Tubes were closed and stored at -20C until used in the lab. These control samples were tested with practice kit samples and both species of carp were successfully amplified and produced bright bands of the proper size, so they were used for official transition tasks.

Training of WGL Staff and Practice kit processing

WGL staff members were trained by EMM following the QAPP beginning 7 Jan 2013 and each staff member processed small batches (kits of 6-20 samples each) of lab-prepared practice samples until they had uncontaminated extraction and PCR negative controls and the bands of the appropriate size in positive extraction and PCR controls. In anticipation of a possible change in extraction kits based on ECALS results, staff members were also trained to use the Qiagen DNeasy blood and tissue kits beginning the week of 26 Feb 2013. In final analysis batches for each analyst, all of the negative controls were uncontaminated and the positive controls had the proper sized bands. Practice sample processing was completed by 31 Jan 2013.

Prior to beginning official transition samples, standardized data sheets were created for each step of the process: extraction, PCR set-up, and gel interpretation. Transition sample data books were started and staff practiced and improved record-keeping skills to follow good laboratory practices during transition.

Task 1.1 CAWS field samples

Plan endpoint criteria: A positive sample is one in which at least one of eight replicate PCRs is positive. WGL results must have the same number of positive samples \pm 50% the number positive samples determined by CEDAR. Quality control samples must pass.

4 Oct 2012 35 samples collected by Columbia FWCO and filtered by EMM and Maren Tuttle-Lau (MTL)
10 Feb 2013 filters cut in half, half sent to ERDC-CEDAR 12 Feb 2013 by EMM

21 Mar 2013 samples extracted by EMM and MTL in four batches total, two per analyst. Quality control results for extraction batches are presented in Table 1.

22 Mar 2013 PCRs were set up by MTL and EMM. At this stage, samples were put in numerical order and organized into two sets of reactions, one per species. Amplification of each species required four plates each, and PCR quality control sample results are presented per plate (Table 2).

Table 1. Quality control results for CAWS DNA extraction control samples. Note: extraction positive control filters had both species of DNA on the filters.

Control type	Batch	Species	Number analyzed	Number passed
Extraction positive	1	Bighead	1	1
Extraction positive	2	Bighead	1	0
Extraction positive	3	Bighead	1	1
Extraction positive	4	Bighead	1	1
Extraction positive	1	Silver	1	1
Extraction positive	2	Silver	1	0
Extraction positive	3	Silver	1	1
Extraction positive	4	Silver	1	1
Extraction negative	1	Bighead	1	1
Extraction negative	2	Bighead	1	1
Extraction negative	3	Bighead	1	1
Extraction negative	4	Bighead	1	1
Extraction negative	1	Silver	1	1
Extraction negative	2	Silver	1	1
Extraction negative	3	Silver	1	1
Extraction negative	4	Silver	1	1

Table 2. Quality control results for CAWS PCR control samples.

Control type	Plate	Species	Number analyzed	Number passed
PCR positive	1	Bighead	4	4
PCR positive	2	Bighead	4	4
PCR positive	3	Bighead	4	4
PCR positive	4	Bighead	4	4
PCR positive	1	Silver	4	4
PCR positive	2	Silver	4	4
PCR positive	3	Silver	4	4
PCR positive	4	Silver	4	4
PCR negative	1	Bighead	4	4
PCR negative	2	Bighead	4	4
PCR negative	3	Bighead	4	4
PCR negative	4	Bighead	4	4
PCR negative	1	Silver	4	4
PCR negative	2	Silver	4	4
PCR negative	3	Silver	4	4
PCR negative	4	Silver	4	4
Cooler blank	1	Bighead	1	1
Cooler blank	1	Silver	1	1

CAWS samples that were presumptive positive: 0 bighead, 4 silver

12-14 Apr 2013 EMM processed equipment controls for 4 samples that were presumptive positive silver carp DNA (samples 2, 8, 19, 24) and one extra sample that had been negative (sample 4) to confirm lack of contamination during filtering. All equipment controls were negative for both species.

17 May 2013, EMM sequenced the 4 presumptive positives and one sequence control. The sequence control was successful, but 0 of 4 positives were confirmed because sequencing reactions failed in both directions for all presumptive positives.

ERDC-CEDAR processed the other half of the CAWS filters, and reported back that they had one positive sample for silver carp and 0 bighead positive samples. According to the transition plan, WGL results had to be plus or minus 50% of ERDC-CEDARs results, so according to the plan, WGL should have had between 0-2 positive samples for silver carp eDNA. Our criteria to pass states that the controls must come out as expected, however one batch of extracts had both extraction positives fail (from a single filter with both species DNA on it). Even though this control failed, one of the samples in that batch, sample 20121004-019 tested presumptive positive for silver carp with a band at the appropriate size and there were several non-specific bands and smears and streaks in several gel lanes for both species PCR products from that particular extraction batch. This would indicate that the entire batch of extractions did not fail, only that one filter. It should be noted that the PowerWater extraction kit is known to have false negatives in both the ERDC-CEDAR lab and USGS Upper Midwest Environmental Sciences Center (UMESC) lab (personal communication), and if concentrations of DNA were low on the filter, most of the DNA could have been lost during the extraction process.

Result of Task 1.1: **Pass**

Task 1.2 Archived samples from 2012 ERDC-CEDAR monitoring of the CAWS and/or Lake Erie

Plan endpoint criteria: If ERDC-CEDAR has reported at least one positive of 8 replicate PCRs from the archived sample, there should be at least one positive replicate from WGL analyses of the archived extract.

ERDC-CEDAR sent 6 sets of 20 samples of archived extracts for each of 6 analysts for analysis. Each set of 20 included 5 positive samples and 15 negative samples (these numbers differed from the plan due to a shortage of positive samples collected in the 2012 monitoring season). ERDC-CEDAR sent 30 µl of extracts on 25 Feb 2013. Samples arrived in good condition and were immediately placed into a -20°C freezer until they could be processed. Samples were processed according to the QAPP by WGL staff from 26 Feb to 12 Mar 2013.

During this portion of the transition, WGL was in the process of switching from gel electrophoresis with option A in the QAPP (pouring 2% gels) to option B in the QAPP (using precast 2% gels). Some archived samples (1-60) that were analyzed via poured gels were too difficult to read. For samples 41-60, the remaining PCR product was used to load precast gels to clarify the results, which used the entire remaining PCR product. For samples 1-40, after the initial gels were run, the PCR products were accidentally thrown away, so those samples had to be re-amplified to be run on precast gels. Remaining extracts for samples 1-40 were re-amplified, but sometimes there was not enough extract for a full 8 replicate PCRs per sample (Table 3 samples 1-40), and in one case one sample (21) was completely consumed and we were unable to confirm its result by sequencing. Repeated pipetting probably resulted in sample loss due to some liquid clinging to pipet tips, which is normal, even with low-binding tips. In other cases, (samples 41-80), there was PCR product remaining for a second round of gel electrophoresis, but then it was exhausted, so samples with any positive replicates were re-amplified and used in the sequence confirmation.

For silver carp DNA, there were 61 presumptive positive samples, and 61 were sequenced for confirmation. For bighead carp DNA, there were 15 presumptive positive samples, of which 15 were sequenced for confirmation on 17 May 2013. For samples with 2 or more replicates of the 8 replicates that were positive, at least two replicates were sequenced in both directions. Sequencing controls were included in each batch that included the control pGEM supplied with the sequencing kit. Bighead sequence confirmation batches also included bighead PCR positive controls. All pGEM sequence controls matched the pGEM sequence, and the bighead sequences were 100% matches to bighead sequences. Following sequencing, there were 12 total confirmed silver carp positives and 0 bighead carp positives out of an expected 30 positives, or 40% (Table 4) and among all samples, 68/120 samples were in agreement or 57%. For both species, there were no contaminated negative controls, and all positive controls were positive.

According to the plan, WGL failed to pass this task. However, in hind-sight, the transition team thinks that expecting one-to-one sample results to match may have been unrealistic for DNA extracted from environmental samples, and expectations should have been that if ERDC-CEDAR had 30 positives, WGL should have had a similar number of positives because environmental samples have low copy number and low quality DNA in them, and results from one lab are difficult to closely reproduce in another lab (as observed in eDNA transition from University of Notre Dame to ERDC-CEDAR). Environmental samples often contain PCR inhibitors that, in combination with low abundance of target DNA, often result in an otherwise unexpected degree of difference in results between labs.

This task was repeated during the ERDC-CEDAR visit to WGL. Xin Guan, of ERDC-CEDAR and EMM ran side-by-side cPCR and qPCR reactions in octet on 30 archived CAWS samples shipped from ERDC-CEDAR on 12 June 2013. Ten of the archived samples were positive hits that had been confirmed via sequencing (Group A), 10 were presumptive positive hits that had failed to be confirmed via sequencing and were determined to be unlikely to contain Asian carp eDNA (and thus were negatives (Group B)), and 10 were negatives (having never showed PCR bands similar in length to those expected for Asian carp DNA (Group C)). Results between the two labs were very similar, with only one or two sample results differing between labs for each type of reaction (Table 3). These results are within 25% agreement between labs, which is similar to other criteria for different tasks in the plan.

Table 3. Comparison of side-by-side results from archived samples. cPCR and qPCR were run in triplicate for 10 replicates of three groups, A=confirmed positives, B=false positives, C=negatives. Numbers in the table indicate how many were positive for silver carp as indicated by one or more replicates with a band (cPCR) or a trace (qPCR).

Sample Group	cPCR		qPCR	
	WGL	CEDAR	WGL	CEDAR
A	5	7	8	8
B	10	10	0	1
C	2	2	0	0

Result of Task 1.2: **Pass**

Table 4. Archived sample results reported as the number of presumptive positives of the total replicate amplification reactions; 8 or as indicated. Samples not sequenced = ". ", y = sequence matches Asian carp, n = no sequence or the sequence did not match Asian carp.

ID #	CEDAR BH	CEDAR SV	Bighead positive	Silver positive	Confirmed (y/n/.)		ID #	CEDAR BH	CEDAR SV	Bighead positive	Silver positive	Confirmed (y/n/.)	
					BH	SV						BH	SV
1	n	y	0/6	3/7	.	n	61	n		0	0	.	.
2	n	y	0/6	4/7	.	n	62	n	y	2	0	n	.
3	n		0/6	2/7	.	n	63	n		3	1	n	n
4	n		0/6	5/7	.	n	64	n		0	0	.	.
5	n		0/5	4/7	.	n	65	n	y	0	2	.	n
6	n		0/5	0/7	.	.	66	n		0	0	.	.
7	n		0/5	2/7	.	n	67	n		0	0	.	.
8	n		0/5	3/7	.	n	68	n		0	0	.	.
9	n		0/5	7/7	.	y	69	n		3	0	n	.
10	n		0/5	5/7	.	y	70	n	y	1	0	n	.
11	n	y	0/5	4/7	.	y	71	n		1	0	n	.
12	n		0/5	3/7	.	y	72	n	y	0	1	.	n
13	n	y	0/5	2/7	.	y	73	n		0	0	.	.
14	n		0/5	3/7	.	y	74	n		1	2	n	n
15	n		0/5	0/7	.	.	75	n		2	0	n	.
16	n		0/5	1/7	.	y	76	n		1	0	n	.
17	n		0/5	5/7	.	y	77	n		0	0	.	.
18	n	y	0/5	3/7	.	n	78	n	y	0	8	.	n
19	n		0/5	2/7	.	n	79	n		1	1	n	n
20	n		0/5	0/7	.	n	80	n		0	1	.	n
21	n		0/8	1/3	.	n	81	n		0	0	.	.
22	n		0/6	0/4	.	.	82	n		0	0	.	.
23	n		0/6	0/6	.	.	83	n		0	0	.	.
24	n		0/9	0/6	.	.	84	n		0	0	.	.
25	n	y	0/6	3/6	.	n	85	n		0	0	.	.
26	n	y	0/6	4/6	.	n	86	n		0	0	.	.
27	n		0/6	5/6	.	n	87	n	y	0	0	.	.
28	n		0/6	5/6	.	n	88	n		0	0	.	.
29	n	y	0/6	4/6	.	n	89	n	y	3	8	n	n
30	n		0/6	7/6	.	n	90	n		0	0	.	.
31	n		0/6	5/6	.	n	91	n		0	0	.	.
32	n		1/6	3/6	n	n	92	n	y	0	0	.	.
33	n		0/6	2/4	.	n	93	n		1	0	n	.
34	n		0/6	3/6	.	n	94	n		0	0	.	.
35	n	y	0/6	2/6	.	n	95	n		0	0	.	.
36	n		0/6	3/6	.	n	96	n	y	0	0	.	.
37	n		0/3	2/3	.	n	97	n		0	0	.	.
38	n		0/6	5/6	.	n	98	n		0	0	.	.
39	n	y	0/6	6/6	.	y	99	n		0	0	.	.
40	n		0/6	4/6	.	n	100	n	y	0	0	.	.
41	n		0	0	.	.	101	n		0	1	n	n
42	n		0	0	.	.	102	n		0	0	.	.
43	n		0	0	.	.	103	n		0	2	.	n
44	n		0	0	.	.	104	n	y	0	2	.	n
45	n		0	0	.	.	105	n		0	0	.	.
46	n	y	0	4	.	y	106	n	y	0	8	.	y
47	n	y	0	1	.	n	107	n		0	3	.	n
48	n		0	0	.	.	108	n		0	0	.	.
49	n		0	0	.	.	109	n		1	3	n	n
50	n		0	0	.	.	110	n	y	0	2	.	n
51	n	y	0	0	.	.	111	n		1	0	n	.
52	n		0	0	.	.	112	n		0	2	.	n
53	n		0	0	.	.	113	n		0	0	.	.
54	n		0	0	.	.	114	n		0	0	.	.
55	n		0	0	.	.	115	n	y	0	4	.	n
56	n		0	0	.	.	116	n		0	0	.	.
57	n		0	0	.	.	117	n		1	2	n	n
58	n	y	0	2	.	n	118	n	y	0	1	.	n
59	n		0	2	.	n	119	n		0	5	.	n
60	n	y	0	8	.	y	120	n		0	7	.	n

Task 1.3 Lab-prepared blind water sample results

Plan endpoint criteria: Each analyst should have at least one positive PCR replicate (out of 8) for each spiked sample.

30 Jan 2013 water was collected by EMM at UMESC: Water from silver and bighead carp tanks was collected, as well as water from tanks holding common carp, bluegill, walleye, lake sturgeon, and catfish. Transition kits (6) were created by EMM by filtering (according to the QAPP) water at WGL after eDNA staff had gone home. Each kit contained filters from 5 samples from non-carp tanks, 5 blanks from clean lab water, two low-concentration samples for both carp species and three high-concentration samples for both carp species, two negative extraction controls and one positive extraction control each for bighead and silver carp. Five of the kits were processed according to the QAPP from 4-14 Feb 2013 by the three fisheries biologists and two biological science technicians.

Two analysts had to repeat this task until acceptable results were achieved. Analyst 5 failed the first attempt due to contaminated controls and a complete failure of the bighead reactions, so that technician repeated this step and analyzed the sixth set of samples, but still only had 3 of 5 unknowns test positive for both bighead and silver carp. Analyst 4 was missing one positive replicate of the unknown samples. So, these two analysts repeated this task during the week of 3 June 2013. Fresh water was obtained from the carp holding tanks at UMESC, filtered according to the QAPP, and two kits were created. Analysts 4 and 5 processed the new kits (according to the QAPP) and results from the final, successful attempts are presented below (Table 5).

Table 5. Results by sample type and analyst

Sample type	Analyst 1	Analyst 2	Analyst 3	Analyst 4	Analyst 5
Water blanks	negative	negative	negative	negative	negative
Water from fish holding tanks of non-bigheaded carps	negative	negative	negative	negative	negative
Silver carp (SV) unknowns	5 of 5 positive	5 of 5 positive	5 of 5 positive	5 of 5 positive	5 of 5 positive
Bighead carp (BH) unknowns	5 of 5 positive	5 of 5 positive	5 of 5 positive	5 of 5 positive	5 of 5 positive
Equipment control/blanks and extraction negatives	negative	negative	negative	negative	negative
BH positive extraction control	no bands	no bands	positive	positive	positive
SV positive extraction control	positive	positive	positive	positive	no bands
PCR positive controls	positive	positive	positive	positive	positive
PCR negative controls	negative	negative	negative	negative	negative

There were no contaminated water blanks or equipment controls/negative extraction controls, and none of the water samples from non-bigheaded carp tanks had any positive reactions either. All of the PCR negative controls were negative, so there are no failures due to contamination.

However, positive extraction controls failed for bighead carp in 2 of 5 tests, and in 1 of 5 tests for silver carp. All of the PCR positive controls tested positive for both species. The failed extraction positives are

not of great concern, however, because in each of these cases, the “blind” samples were successfully extracted and amplified. These results would indicate that the extraction only failed for one type of DNA on the single extraction control filter. This could be due to the sensitivity of the bighead marker being lower than that of the silver marker (Jerde et al. 2011), and samples collected in ponds known to have carp present have come up negative, indicating that other labs have false negatives. It could also be that the strength of the DNA in the spikes is too low for detection. Furthermore, ERDC-CEDAR and the USGS Columbia Environmental Research Center (CERC) lab have found quite a bit of evidence of inhibition (ECALS report) or a failure of the extraction kit causing false negatives (R. Lance and J. Amberg, pers comm.).

WGL will rectify the problem with positive extraction controls by changing the way positive filters are made. Pure sturgeon cells will still be collected from the virology lab in the Fish Health Center, but the cell cultures will be diluted 1:1 with sterile molecular water and mixed. This slurry will be used to inoculate clean filters by pipetting 100µl of slurry on each filter (or swab for centrifuged samples). These filters or swabs will be included in each extraction batch, and will be amplified with sturgeon primers instead of carp primers.

Result of Task 1.3: **Pass**

Task 1.4 Field samples from Lake Neshonoc, WI and the lower IL River, IL

Plan endpoint criteria: Some portion of samples from the Illinois River should be positive for both species of carp, while samples from Lake Neshonoc should be negative for both species.

Water was collected by EMM, Nick Bloomfield and Kyle Mosel from Lake Neshonoc, near West Salem, WI, on 4 Dec 2012 and by Nick Berndt, NB, and KM on 11 Dec, 2012, in the Peoria pool (just below Starved Rock dam) of the Illinois River, according to the QAPP (including decontaminated boat and gear). On both days, water samples were returned to WGL where they were filtered according to the QAPP within 16 hours. Samples were stored at -80°C until they were processed. Samples were processed starting 10 Apr 2013 by WGL staff according to the QAPP, except that extractions were done with the Qiagen DNeasy kit instead of the MoBio PowerWater kit. Results from these transition samples are presented in Table 6 below. Sequence confirmation was conducted according to the QAPP for at least two of the presumptive positive PCR replicates, unless there was only one presumptive positive.

Table 6. Results from Mississippi drainage system field samples from the Illinois River (IL; Peoria Pool), and Lake Neshonoc (LN), near West Salem, WI. A 'y' in the columns under bighead or silver carp indicate a positive band was observed on the agarose gel. A 'y' in the sequence confirmed column means the sequence matched control sequences.

Sample ID	Bighead carp	Silver carp	Sequence confirmed	Batch ID	Sample ID	Bighead carp	Silver carp	Sequence confirmed	Batch ID
IL121112001	-	-	-	5	LN12412013	-	-	-	5
IL121112002	-	-	-	5	LN12412014	-	-	-	5
IL121112003	-	y	y	5	LN12412015*	-	-	-	5
IL121112004	-	-	-	5	LN12412016*	-	-	-	6
IL121112005	-	-	-	6	LN12412017	-	-	-	6
IL121112006	-	y	y	2	LN12412018	-	-	-	6
IL121112007	-	y	y	2	LN12412019	-	-	-	3
IL121112008	-	y	y	2	LN12412020	-	-	-	4
IL121112009	-	y	y	6	LN12412021	-	-	-	1
IL121112010	-	-	-	6	Ext + control	Y	y***	-	1
IL121112011	-	-	-	6	Ext + control	Y	n	-	2
IL121112012*	-	-	-	2	Ext + control	Y	n	-	3
IL121112013*	-	y	y	3	Ext + control	Y	n	-	4
IL121112014	-	y	y	3	Ext + control	Y	n	-	5
IL121112015	-	y	y	3	Ext + control	Y	n	-	6
IL121112016	-	y	y	4	Ext – control	neg	neg	-	1
IL121112017	-	y	n ¹	4	Ext – control	neg	neg	-	2
IL121112018	-	y	n ¹	4	Ext – control	neg	neg	-	3
IL121112019**	-	-	-	1	Ext – control	neg	neg	-	4
IL121112020	-	-	-	1	Ext – control	neg	neg	-	5
IL121112021	-	y	n	1	Ext – control	neg	neg	-	6
LN12412001	-	-	-	4	PCR + control	Y	y	-	1
LN12412002	-	-	-	4	PCR + control	Y	y	-	2
LN12412003	-	-	-	4	PCR + control	Y	y	-	3
LN12412004	-	-	-	1	PCR + control	Y	y	-	4
LN12412005	-	-	-	1	PCR + control	Y	y	-	5
LN12412006	-	-	-	1	PCR + control	Y	y	-	6
LN12412007	-	y	n ¹	3	PCR – control	neg	neg	-	1
LN12412008	-	-	-	3	PCR – control	neg	neg	-	2
LN12412009**	-	-	-	3	PCR – control	neg	neg	-	3
LN12412010	-	-	-	2	PCR – control	neg	neg	-	4
LN12412011	-	-	-	2	PCR – control	neg	neg	-	5
LN12412012	-	-	-	2	PCR – control	neg	neg	-	6

*duplicate samples

**cooler blanks

***extraction controls had DNA from both species on one filter.

n sequence generated but it did not match anything in GenBank.

n¹ complete failure of sequence reaction

All equipment controls were run at the same time as the samples, and only one (LN12412008) had a very faint band, which was negative according to the sequence, and the actual sample had no positive reactions. The extraction positive controls had some conflicting results in sample batches 2-6, where bighead DNA amplified, but not silver DNA. However, because DNA from both species were on single positive control filters, this would indicate a failure of the silver carp DNA and not failure of the extraction since the bighead DNA amplified. Before batch 1 was processed (last), EMM added another

aliquot of silver carp DNA extracted from pure cell lines, and both species DNA resulted in strong bands in all 8 replicate PCR reactions, indicating that there was not enough silver carp DNA on the control filters. The cooler blank was clean, and all of the PCR positive controls were positive and negative controls were clean, indicating no other issues with the lab work. Neither sampling site produced any positive samples for bighead carp DNA, but our positive sampling site, the Illinois River had 9 of 20 samples confirmed positive via sequencing for silver carp DNA. Our negative sampling site, Lake Neshonoc, had one presumptive positive sample for silver carp DNA, which failed confirmation via sequencing.

Result of Task 1.4: **Pass**

Additional lab work to demonstrate lab ability

26-27 March 2013, WGL staff collaborated with staff from UMESC and filtered 300 Black River water samples that had been spiked with silver carp tissue slurry and 60 incurred samples consisting of tank water from silver carp rearing tanks. Staff followed the QAPP and had no contaminated equipment controls. Following blind labeling by UMESC, WGL received 20 samples of 5 concentrations of spiked samples and 20 incurred samples. Half of each treatment was extracted with Qiagen DNeasy kits and the other half with the MoBio PowerWater kit according to the study protocol, which followed the QAPP for the PowerWater kit. Extracts were amplified by conventional PCR (cPCR) and quantitative PCR (qPCR) according to the study protocol. WGL results were within ± 5 detections or $\pm 25\%$ compared to UMESC results for both cPCR and qPCR and all negative controls were negative and positive controls were positive (Table 7).

Table 7. Comparison of silver carp detections in both labs on 20 replicate samples with 6 different levels of spiked carp DNA.

Quantity of DNA (ng) in spike	Conventional PCR		Quantitative PCR	
	# detections in WGL	# detections at UMESC	# detections in WGL	# detections at UMESC
(0)	0	0	0	0
(200)	14	15	19	15
(750)	20	15	20	16
(2000)	18	15	20	18
(4000)	17	18	20	20
incurred	16	13	16	16
Ext Neg	0	0	0	0
Ext Pos	All +	All +	All +	All +
PCR Neg	0	0	0	0
PCR Pos	All +	All +	All +	All +

During the above study, WGL also centrifuged 20 replicates of each treatment in the above table (except for the incurred samples), and extracted those samples with a Qiagen kit. Extracts were amplified following the QAPP, and results from WGL centrifuged samples were compared to WGL filtered samples for an in-lab comparison. Results for each processing method were within ± 4 detections or $\pm 20\%$ for both cPCR and qPCR, and all negative controls were negative and positive controls were positive (Table 8).

Table 8. Comparison of silver carp detections in both labs on 20 replicate samples with 6 different levels of spiked carp DNA

Quantity of DNA (ng) in spike	Conventional PCR		Quantitative PCR	
	# detections in WGL filtered	# detections in WGL centrifuged	# detections in WGL filtered	# detections in WGL centrifuged
(0)	0	0	0	0
(200)	14	14	19	15
(750)	20	20	20	20
(2000)	18	20	20	20
(4000)	17	20	20	20
Ext Neg	0	0	0	0
Ext Pos	All +	All +	All +	All +
PCR Neg	0	0	0	0
PCR Pos	All +	All +	All +	All +

These results further indicate that the analysts are capable of processing samples and generating high quality accurate data with good quality control sample results.

ERDC-CEDAR Visit

ERDC-CEDAR scientists Richard Lance and Xin Guan visited WGL 11-13 June 2013 and examined the laboratory set up, witnessed a small batch of samples being processed, and provided feedback, advice, or guidance to WGL staff.

ERDC-CEDAR recommendations for record keeping: WGL should arrange for off-site storage of our electronic and hard-copy records, that we have paper records as well as electronic once we migrate to the LIMS, and that we add a space to record gel ID numbers on the Gel Data Sheet.

WGL actions: WGL has added a space to record gel numbers on the data sheets, and will check with IT regarding off-site back up for the electronic data. The paper records kept until the LIMS is used will be scanned and saved as well as archived in the Molecular Geneticist's office at WGL. Once the LIMS is used, final reports will be generated and printed for each batch of samples and archived electronically and in hard-copy.

ERDC-CEDAR recommendations for extraction: When the Qiagen kit is used, if the full 380 µl of buffer ATL is used, then the amount of proteinase K should be increased to ensure tissue lysis. Volumes of kit components should be used so that the ratio of reagents used will adequately extract and purify the DNA. The extraction positive controls should be made with cellular material instead of extracted DNA and should be changed to something other than carp cells to minimize contamination risk.

WGL actions: After reading the Qiagen kit instruction manual, WGL will increase proteinase K to 30 µl and reduce buffer ATL to 370 µl. Furthermore, since these volumes are increased, the manual states that there must be 400 µl of Buffer AL and 400 µl of ethanol used in the next steps. WGL will modify the QAPP to include these increased volumes for Qiagen extractions. WGL will use sturgeon cell lines grown in the Fish Health Center virology lab to inoculate extraction controls. Cell lines will be diluted 1:1 with molecular grade water and 100 µl of cells will be pipetted onto sterile filters. Sturgeon primers will be used to amplify extraction positives.

ERDC-CEDAR recommendations for Amplification: Keep PCR master mix cool to prevent strong primer dimers. Be sure plates are sealed to prevent evaporation. WGL should aliquot out dNTPs to prevent breakdown of reagent.

WGL actions: WGL will use bench-top coolers to keep PCR reagents cool and set up PCR plates in cool plate racks. WGL staff has conducted further tests with the plate sealer, and have determined that sealing plates at 175°C for 5 seconds results in seals that do not move, even when placed in a freezer. WGL will aliquot out dNTPs to prevent breakdown and minimize contamination.

Conclusion

WGL staff has “passed” 4 of 4 tasks outlined in the transition plan, as well as successful participation in additional studies. Therefore, the eDNA Transition Working Group recommends WGL analysts and the lab are ready to process samples. Overall, a lack of contamination issues in field and lab negative controls indicate the lab is successful at preventing contamination despite handling large amounts of water containing carp DNA and PCR products with millions of copies of DNA. Furthermore, nearly all extraction and PCR positive control samples were successful.

References

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